Reference: *Biol. Bull.*, **152**: 406–414. (June, 1977)

ISOLATION OF NEMATODE INHIBITOR FROM HEMOLYMPH OF THE SNAIL, *HELIX ASPERSA*

CHARIYA RATANARAT-BROCKELMAN[1]

Laboratory of Parasitology, The Rockefeller University, New York, New York 10021

Natural resistance of molluscan hosts to the invasion of parasitic trematodes and nematodes, involving both cellular and humoral responses, has been reported and reviewed extensively (Tripp, 1963, 1974; Acton, 1970). The presence of biologically active material in gastropod hemolymph which has an inhibitory effect on maturation and reproduction of a nematode symbiont has been reported recently (Ratanarat-Brockelman, 1975). This symbiotic relationship involves low infestations (2–8 third stage larvae) of *Rhabditis maupasi* in the mantle cavity of the food snail *Helix aspersa*. Mechanical expulsion of worms occurs when higher levels of infestation are made, suggesting two possible regulatory mechanisms: competition among nematodes, or host control over the level of infestation.

Although the worm was found free in the mantle cavity of the host in experimental infections, other, naturally-infected, snails have been found with worms within the visceral mass as well (G. J. Jackson, personal communication). Evidently the worms can penetrate the cavity wall, and perhaps routinely irritate or rupture the wall in anchoring and feeding, thus exposing themselves to the inhibitor. This aspect of the relationship, as well as the precise source of nutrition of the worms, needs additional clarification.

Third stage larvae that became established in the snail's mantle cavity did not undergo molting or morphological development until the snail died. The nematode larvae then began to grow, molted twice, became sexually mature and reproduced prolifically until the snail tissue was completely consumed. At this time they entered the soil provided, and were capable of reinfesting a new host.

An inhibitor which retards development and reproduction of worm populations cultivated *in vitro* was isolated from fresh snail plasma (Ratanarat-Brockelman, 1975). This inhibitor interfered with larval development and with differentiation of the reproductive organs in maturing worms. The action of the inhibitor varied directly with concentration, and it was found to have two complementary parts: a diffusable cofactor and a proteinaceous component. The present report presents further results on the analysis of the proteinaceous component.

Materials and Methods

Nematode

Specimens of *Rhabditis maupasi* were grown axenically on agar slant cultures. The offspring had been subcultured continuously since 1972. A mixed population

[1] Present address: Department of Microbiology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 4, Thailand.

of the nematode was harvested from an agar culture one day before each bioassay and suspended in 5% sterile dextrose solution. Third stage larvae were separated from other stages in a neo-Baermann apparatus (Ratanarat-Brockelman and Jackson, 1974) at 20° C. The larvae which migrated out were collected in 5% dextrose solution for inoculation into test cultures.

Snail plasma

The specimens of *Helix aspersa* used for bleeding in this report were originally obtained from a fish market in Bronx, New York. Half of each lot purchased was dissected and examined for helminthic infection. The remainder were kept in aestivation at 21° C. Before the snails were used in experiments, they were rehydrated in distilled water and allowed to feed on oatmeal.

Hemolymph was collected from 300 uninfected snails by making an incision along the *vena magna* and pipetting the hemolymph out until the bleeding stopped. After this procedure, the snails were partially shelled, making a second incision possible in the visceral sac where hemolymph was again collected and pooled to 300 ml volumes. Gelatinous materials (including free cells and the respiratory pigment, hemocyanin, accompanying hemolymph during collection) were separated from the plasma by refrigerated ultracentrifugation in a Beckman Model L preparative ultracentrifuge at $78{,}000 \times g$ for 2 hr. The plasma which remained as supernatant was pooled and then concentrated by pressure filtration through Visking cellulose tubing (casing size 8DC) as described by Craig (1968) ; 50 ml of plasma was concentrated to 10 ml in four hr at 4° C. After concentration, the filtrate containing the co-factor was collected, sterilized by Seitz filtration and stored at 4° C for biassay (Ratanarat-Brockelman, 1975). The plasma concentrate retained in the tubing is referred to as "nondiffusable" since the molecular weights of the components were all higher than 10,000 (Craig, 1968). This concentrate was sterilized by Seitz filtration and refrigerated.

Isolation of inhibition

Separation by ion exchange chromatography. The concentrated snail plasma was dialyzed in a tube of Diaflow UM 3 membrane against cold potassium phosphate buffer of ionic strength 0.15 M and pH 7.8, and applied to a chromatographic column (4.0 × 60 cm) of DEAE-A25 Sephadex. The sample volume at each run was 15 ml and contained 60 mg of protein. It was eluted at 4° C employing an elution schedule of a linear salt gradient (King, 1968). The gradient was produced by connecting two buffer vessels. The starting buffer, connected to the column, was potassium phosphate (pH 7.8, ionic strength 0.15 M). The second vessel, connected with the first one at the bottom, was filled with limiting buffer whose salt concentration was 0.6 M NaCl. The flow rate of buffer was 40 ml per hr. Fractions of 3 ml each were collected and the protein content was determined spectrophotometrically at 280 nm. All protein-containing fractions were pooled to nine portions according to optical density and concentrated in boiled and prechilled Visking cellulose tubing (23/32 in diameter) against polyethyleneglycol, mol. wt. 6000, which reduced the volume from 60 ml to 5 ml. The concentrated fractions were dialyzed against 0.85% NaCl overnight and assayed for protein

content by the method of Lowry, Rosebrough, Farr, and Randall (1951). They were sterilized by Millipore membrane filtration type Swinnex GS-13. Most procedures were carried out at 4° C.

Gel filtration. Gel filtration was done on columns of Sephadex G-200. The packed columns of 1.6 × 120 cm were washed and eluted with 0.15 M potassium phosphate buffer, pH 7.8. Void volumes, determined by passing 0.2 ml of 1% Blue Dextran (Pharmacia, Uppsala, Sweden) dissolved in the same buffer through the column, were found to be about 57 ml. These columns were to fractionate the active protein obtained from the cellulose ion exchanger.

Two ml of inhibitor (2.0 mg protein) was fractionated at a time by gel filtration in potassium phosphate buffer at a flow rate of 12 ml per hr. Individual fractions of 2.6 ml were collected and determined spectrophotometrically for protein content at 280 nm which gave a density profile with three peaks. The three protein portions were again concentrated individually to 2 ml, dialyzed in 0.85% NaCl, and sterilized by membrane filtration.

Starch zone electrophoresis. Purity of the isolated inhibitory protein was examined by starch zone electrophoresis (Kunkel and Slater, 1952; Dusanic and Lewert, 1963) in a veronal buffer of pH 7.8 with an ionic strength of 0.1 M. For each run, 2 ml of sample containing 400 μg of inhibitor was applied to a 50 × 4 × 0.7 cm starch block. The electrophoretic procedure, at 4° C and at 300 volts, was stopped after either 36, 38, or 72 hrs. Each block was partitioned into 1 cm segments. Each segment was eluted with 5 ml of veronal buffer and tested for protein content in individual aliquots (Lowry *et al.*, 1951).

Qualitative determination of the purified factor

Biochemical compounds which usually accompany protein were determined. Montgomery's test (1957) was used to examine for the presence of glycogen. Presence of nucleic acid was searched for by precipitation in 0.2 N acetic acid followed by safranin staining (Rapoport and Raderecht, 1962). The inhibitor was also placed in ether and chloroform to test its solubility, which would give a primary indication of lipid.

Bioassay technique

Protein concentrations of all fractions were quantified by the method of Lowry *et al.* (1951). Dilutions for experimental assays were adjusted with normal saline to equalize the concentration at 1 mg per ml, then mixed 1 : 1 with hemolymph diffusate. One-ml volumes of this were distributed (six replicates per protein portion) to 25-ml Delong culture flasks which contained the following components: 1 ml of basal Pfahnstiel peptone broth (Pf broth), 0.5 ml raw rabbit liver extract (RLE) containing 30 mg protein per ml, and 0.5 ml of 5% dextrose suspension of about 46 larvae (range 37–59). Thus, the final concentration of snail inhibitory protein was 166.7 μg per ml of culture medium. The control group received 1 ml of 0.85% NaCl solution in place of snail plasma protein. Culture flasks were kept in the dark while being shaken at 40 strokes per minute at 21° C to promote aeration.

Evaluation

The nematode population in each flask was counted microscopically on day 7 over a grid, and on day 14 by dilution and volumetric sampling.

Because of the inhibitor, the rates of population increase differed in the various groups of culture media, and thus they could be used as a bioassay of the activity of the inhibitor (Ratanarat-Brockelman, 1975). Population growth in all cultures during at least the first two weeks was approximately exponential. The rate of population increase during an interval of 14 days could be calculated from the equation $N_{(14)} = N_{(0)}e^{14r}$, where N is the number of worms. Differences among mean rates of increase were tested by analysis of variance.

Results

Isolation by ion-exchange chromatography

A typical elution pattern of inhibitory protein is shown in the ion-exchanger profiles of Figure 1, showing nine major peaks of proteins. These nine protein fractions were bioassayed on nematode populations, each fraction with six replicates. As is illustrated in Figure 2, protein fraction 3, eluted at 0.34 M NaCl gradient (elution volume 770 to 830 ml), resulted in the lowest rate of population increase (0.151/day), which was significantly lower than that of the control cultures receiving no snail plasma protein (0.254/day) $F_{8,\,50} = 7.04$, $P < 0.05$ (see Table 1). Populations receiving fractions 2 and 7 also exhibited lower mean rates

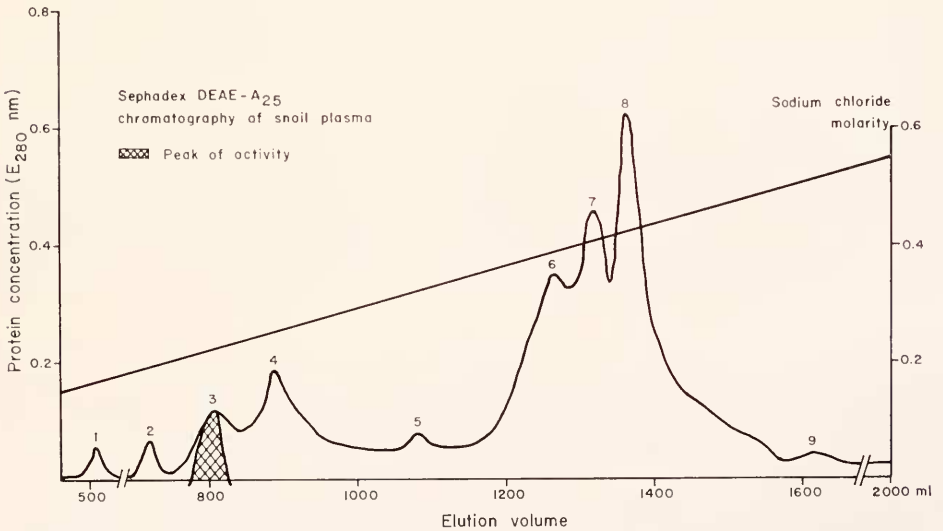

Figure 1. Separation of plasma of *Helix aspersa* on cellulose ion-exchanger, Sephadex DEAE-A 25. Column dimension is 4.0×60 cm; sample volume, 15 ml (60 mg protein); potassium phosphate buffer, 0.15 M, pH 7.8 salt gradient 0.6 M NaCl, flow rate 40 ml/hr, fraction volume 3 ml. All steps were performed at 4° C. Shaded area designates peak of activity.

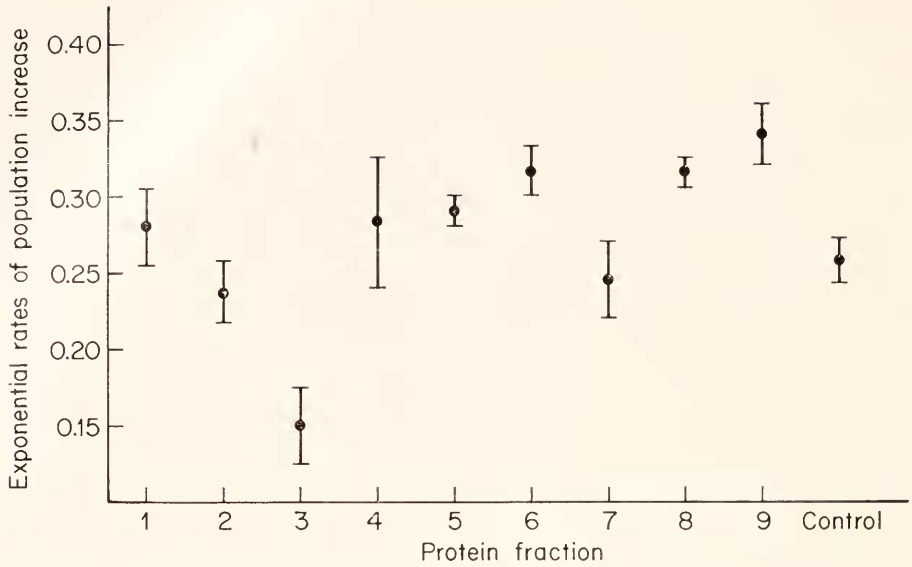

FIGURE 2. Exponential rates of population increase (r) of *Rhabditis maupasi* in nine groups of culture receiving nine different protein fractions separated by cellulose ion-exchanger, Sephadex DEAE-A 25. Time of cultivation was 14 days. Vertical lines represent standard deviations.

of increase (0.238/day and 0.245/day respectively) when compared with that of the control group, but the differences were not significant. Protein peak 3 thus contained most of the inhibitory effects.

Results from the bioassay are further illustrated in Figure 3 which shows log population sizes of nematodes against time. For clearer presentation the 60 cultures were pooled into three groups as follows. One group contained the six control cultures, which showed an average growth of population to $\log_{10}N = 3.04$ (s.d. $\pm$ 0.11) within 14 days. A second group of six cultures receiving protein fraction 3 showed suppression of population growth to $\log_{10}N = 2.23$ (s.d. $\pm$ 0.29). A third group of cultures receiving the remaining fractions, 1, 2, 4, 5, 6, 7,

TABLE I

Analysis of variance of Rhabditis maupasi *population growth rates from nine groups of cultures. Each group (six replicates) received one of the nine protein fractions obtained by fractionation of snail hemolymph protein on an ion-exchange column.*

Source of variation	d.f.	Mean square	F	*P*
Among treatments	9	188.96	4.82	<0.001
Control *vs.* nine expt. groups	1	101.78	2.59	NS
Among nine fractions	8	276.13	7.04	<0.05
Error	50	39.19		
Total	59	66.44		

8, and 9 (each with six replicates), when pooled, showed the highest mean population size of the nematodes ($\log_{10}N = 3.30$, s.d. $\pm$ 0.37).

Fractionation by gel filtration

The further step of fractionating the inhibitory protein on Sephadex G-200 yielded three more peaks eluted at 50, 78 and 150 ml, designated peak 3a, 3b, and 3c, respectively (Fig. 4). The results of bioassay are summarized in Table II, which shows that complete inhibition of reproduction occurred in the cultures receiving protein peak 3c. Although 68.2% of the initial inoculum showed sexual differentiation, in which bursae could be recognized in male worms, the reproductive organs of both sexes remained poorly developed. In male worms, the testes attained only a narrow, straight tubular shape with no coiling. The germ cells were sparse and stained poorly with 1% neutral red solution, suggesting impaired spermatocyst production. Ovaries, uteri and vulva developed in females but contained no eggs. Malfunction of the sexual organs evidently curtailed

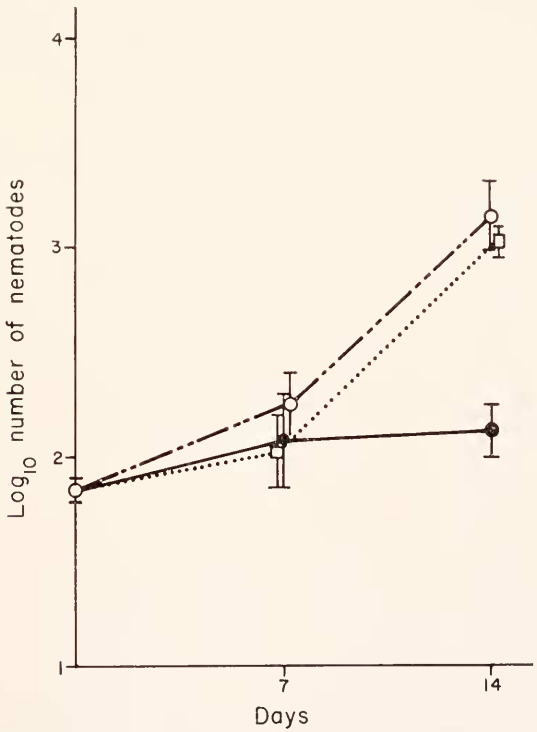

FIGURE 3. Bioassay of different fractions of hemolymph plasma on populations of *Rhabditis maupasi* grown *in vitro*. The three groups of populations shown consist of: first, means of 48 cultures receiving protein peaks, 1, 2, and 4 to 9, (open circles with broken line); secondly, cultures receiving protein peak 3 (solid circles, with full line); and thirdly, control cultures (squares, with dotted lines). Each treatment had six replicates. Vertical bars are standard deviation of log population size.

TABLE II

Rate of Rhabditis maupasi *population growth in three groups of cultures receiving separated protein fractions from Sephadex G-200. Control received only raw rabbit liver extract (four replicates).*

	Control	Cultures receiving fraction		
		3a	3b	3c
Rate of population growth (r)	0.114	0.109	0.070	0.001
Standard deviation	0.06	0.01	0.01	0*
Per cent maturity	100	100	95	68.2**

* Only two larvae were produced in one culture.

** 14.8% remained undeveloped, and 17% developed to stage 4 larvae. There were significant differences in population growth rates among fractions ($F_{2,12} = 8.34$, $P < 0.05$).

offspring production during the 14 days of cultivation, whereas two generations were born to the nematodes in control groups.

The rates of population increase in nematode cultures receiving the two other protein fractions were significantly higher than the group receiving protein peak ($F_{2,12} = 8.34$, $P < 0.05$). Thus, protein peak 3c evidently contained most of the inhibitor.

Purification of inhibitory protein

After the initial step of purification by rechromatographing the active peak 3c alone on a column of Sephadex G-200, the sample obtained was examined

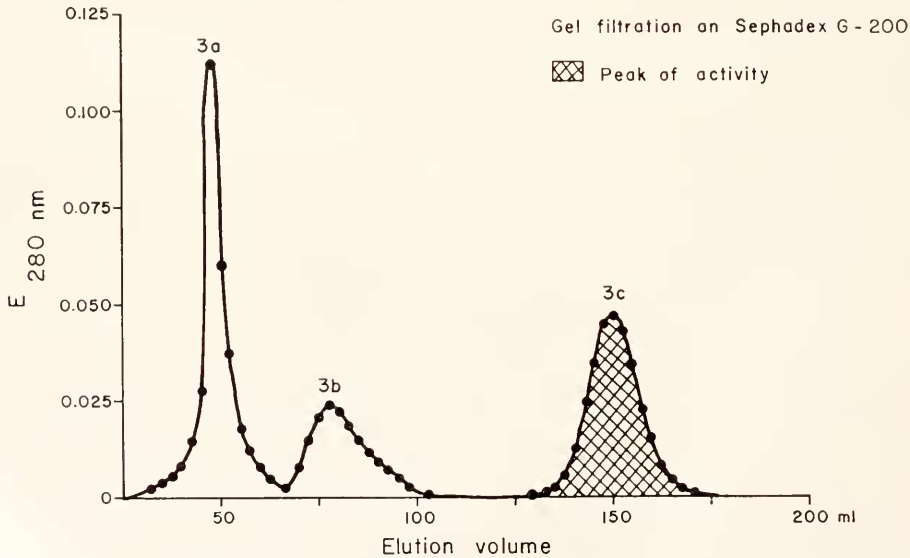

FIGURE 4. Gel filtration of active peak on a column of Sephadex G-200. Column dimension is 1.6 × 120 cm; sample size, 2 ml (2.0 mg protein); potassium phosphate buffer, 0.15 M, pH 7.8; flow rate, 12 ml/hr; fraction volume, 2.6 ml.

electrophoretically on a starch bed. Only one band appeared at each run, suggesting purity of the sample.

Determination of accompanying substances

Neither the test for the presence of glycogen nor that for nucleic acid was positive. The inhibitor did not dissolve in the fat solvents ether and chloroform, suggesting the absence of a lipid component. The inhibitor reacted with the folin-phenol reagent of Lowry *et al.* (1951) and thus appears to be protein.

Discussion

The effects of a hemolymph factor occuring in *Helix aspersa* on the maturation of the nematode *Rhabditis maupasi* have been described recently by Ratanarat-Brockelman (1975). In that report, experimental results suggested a proteinaceous nature of the factor. The finding led to the present investigation, especially intended to establish whether this substance is identical to protectin, an agglutinin of glycoprotein nature which has been found to have a protective function in *Helix pomatia* (Prokop, Uhlenbruck, Rothe, and Cohen, 1974). However, the isolated and purified hemolymph factor reported here was characterized as to protein, not glycoprotein, as the Montgomery test (Montgomery, 1957) for glycogen gave a negative result. Further characterization of the inhibitor will have to await more extensive biochemical work.

The *in vitro* cultivation experiments, using growth media alone and in combination with the inhibitor, illustrated that the inhibitor suppressed the normal function of the reproductive organs but not growth. Although reproductive organs developed in some of the worms, fertilization and reproduction scarcely occurred even in the presence of ample nutrients. The purified factor allowed only 68.2% of the larvae to become sexually mature, although the protein concentration used was only 166 g/ml medium, compared to the much higher concentration required (at least 10 mg) when crude snail extract was used (Ratanarat-Brockelman, 1975). The effect of the inhibitor is not to destroy the nematodes entirely; instead, it permits a commensal relationship between the nematode larvae and the molluscan host with minimal (if any) damage to the host, and helps regulate the population level of the worm.

A full understanding of the significance of this inhibition must include a probable or at least plausible evolutionary explanation of the relationship. I know of no field work on natural populations of these species, but field observations of other *Rhabditis* species in other pulmonates yield a general life history consistent with my laboratory observations (see Filipjev and Schuurmans Stekhoven, 1941; Mengert, 1953; Ratanarat-Brockelman and Jackson, 1974). A reasonable hypothesis is that the inhibitor evolved as a host mechanism to help prevent internal infection of the snail. The worms then evolved a capability of living mostly or completely outside the host tissue and reacting to the disappearance of the inhibitor at the host's death as a cue to resume development and reproduce. It seems unlikely that the nematode could not evolve a chemical counter-defense against such a low concentration of inhibitor if there were selective pressure to do so, if this were the snail's only available defense. The commensal worm thus still gains a large

ready food supply at the death of the snail, a means of dispersal, and a relatively stable moist environment.

This investigation was supported by a Postdoctoral Fellowship from the U. S. National Institutes of Health, grant AI-00192–12. The author wishes to thank Drs. A. Kilejian and F. Brohn for their kind suggestions for biochemical techniques, and Dr. W. Trager for his valuable comments on research approaches and on the preparation of the manuscript.

Summary

Hemolymph plasma of the snail *Helix aspersa* which inhibits maturation and reproduction of its mantle cavity-inhabiting nematode, *Rhabditis maupasi,* was separated biochemically for the active proteinaceous component.

Isolation of the active inhibitor was performed using ion-exchange chromatography in combination with subsequent gel filtration. The isolated peaks were bioassayed *in vitro* on nematode larvae. The fractions harboring inhibitory protein suppressed larval growth and adult reproduction *in vitro.*

The isolated fraction was purified by gel filtration and characterized on the basis of a single band on starch zone electrophoresis and positive reaction only with folin-phenol reagent.

LITERATURE CITED

Acton, R. T., 1970. *Immunobiological and immunochemical studies of the oyster Crassostrea virginica.* Manuscripts Information Corp., New York, 134 pp.

Craig, L. C., 1968. Ultrafiltration. Pages 129–131 in C. Williams Jr., and M. W. Chase, Eds., *Methods in immunology and immunochemistry, Vol. II.* Academic Press, New York.

Dusanic, D. E., and R. M. Lewert, 1963. Alterations of proteins and free amino acids of *Australorbis glabratus* after exposure to *Schistosoma mansoni* miracidia. *J. Infect. Dis.,* **122**: 243–247.

Filipjev, I. N., and H. H. Schuurmans Stekhoven, Jr., 1941. *A manual of agricultural helminthology.* E. J. Brill, Leiden.

King, T. P., 1968. Cellulose ion exchange chromatography. Pages 154–162, in C. Williams Jr., and M. W. Chase, Eds., *Methods in immunology and immunochemistry, Vol. II.* Academic Press, New York.

Kunkel, H. G., and R. J. Slater, 1952. Zone electrophoresis in a starch supporting medium. *Proc. Soc. Exp. Biol. Med.,* **80**: 42–46.

Lowry, H. H., N. J. Rosebrough, H. L. Farr, and R. J. Randall, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.

Mengert, H., 1953. Nematoden and Schnecken. *Z. Morphol. Tiere,* **41**: 311–349.

Montgomery, R., 1957. Determination of glycogen. *Arch. Biochem. Biophys.,* **67**: 378–386.

Prokop, O., G. Uhlenbruck, A. Rothe, and E. Cohen, 1974. Protectins: past, present problems and perspectives. *Ann. N. Y. Acad. Sci.,* **234**: 228–231.

Rapoport, S. M., and H. J. Raderecht, 1962. Nachweis der Nukleinsäuren. Page 133–135 in *Physiologisch chemisches Praktikum.* VEB Verlag Volk und Gesundheit, Berlin.

Ratanarat-Brockelman, C., 1975. Inhibition of *Rhabditis maupasi* (Rhabditidae; Nematoda) maturation and reproduction by factors from the snail host, *Helix aspersa. J. Invert. Pathol.,* **25**: 229–237.

Ratanarat-Brockelman, C., and G. J. Jackson, 1974. *Rhabditis maupasi:* occurrence in food snails and cultivation. *Exp. Parasitol.,* **36**: 114–122.

Tripp, M. R., 1963. Cellular responses of mollusks. *Ann. N. Y. Acad. Sci.,* **113**: 467–474.

Tripp, M. R., 1974. Molluscan immunity. *Ann. N. Y. Acad. Sci.,* **234**: 23–27.